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CYTEC

Product Sustainability & Regulatory Affairs
5 Garret Mountain Plaza
Woodland Park, NJ 07424

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February 22, 2013

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U.S. Environmental Protection Agency
Attn: TSCA Section 8(e) Coordinator
Office of Pollution Prevention and Toxics (OPPT)
1200 Pennsylvania Avenue, NW
Washington, DC 20460-0001

8EHQ-12-18874

ATTENTION: MS. LAVERNE JONES; FAX# 202-564-8955

REFERENCE: 8EHQ-12-18874

Dear Sir/Madam:

In a previous letter dated January 18th, Cytec submitted the final report entitled "Daphnia sp., 48-Hour Acute Immobilization Test" on a mixture identified by Chemical Abstracts Service Registry Numbers ("CASRN") 2956-12-9 and 1311195-87-5 which are described by the chemical names Carbonodithioic acid, O-pentyl S-propenyl ester and Carbonodithioic acid, O-(2-methylbutyl) S-2-propen-l-yl ester, respectively. At that time was Cytec was holding information CBI – but upon further review and confirmation this claim cannot be substantiated – therefore, I am enclosing a new final report marked as non-CBI for your reference.

The enclosed report **does not** contain confidential business information.

If you have any questions or comments please contact me at (973) 357-3375.

Sincerely,

Patricia Ann Vernon
Senior Manager, Global Product Regulatory Compliance

8913000002 92

CONTAINS NO CBI

352341

FAX COVER SHEET

Toxicology & Product Regulatory Compliance Department
If there are problems with this transmission, please call (973) 357-3369

SUBJECT: CBI Claim

MESSAGE:

Dear Ms. Jones -

Please find the second of two letters regarding our previous CBI claim dated February 4, 2013. We are dropping our claim for CBI. Official copy of the letter and non-CBI report is being sent Federal Express along with first letter (report). I will be out of the office M-W next week but can be reached @ 201-314-5179 (cell phone) if you have any questions.

Best regards,

Patti Vernon

TO: Ms. LAUERNE JONES	FROM: Patti Vernon
COMPANY: USEPA TSCA 8(e)	COMPANY: Toxicology/Cytec West Paterson
FAX NUMBER: 202-564-8955 PHONE NUMBER:	FAX NUMBER: (973) 357-3057 PHONE NUMBER: (973) 357-3375
DATE: 2/22/2013	PAGES INCLUDING COVER: 2

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Sincerely,

Patricia Ann Vernon
Senior Manager, Global Product Regulatory Compliance

REPORT

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harlan®

S-10821:
Daphnia sp., 48-Hour Acute Immobilization Test

Data Requirements/Test Guidelines:

- OECD Guidelines for Testing of Chemicals (April 2004) No 202, "*Daphnia* sp., Acute Immobilisation Test".
- Method C.2 of Commission Regulation (EC) No. 440/2008.

Study Director:	S J Parr
Test Facility:	**Harlan Laboratories Ltd.** Shardlow Business Park Shardlow Derbyshire DE72 2GD UK
Sponsor:	**Cytec Industries Inc** Five Garret Mountain Plaza Woodland Park New Jersey 07424 United States of America
Harlan Study Number:	**41203136**
Study Completion Date:	17 January 2013

CONTENTS

STUDY DIRECTOR STATEMENT OF GLP COMPLIANCE

Harlan Laboratories Ltd., Shardlow Business Park, Shardlow, Derbyshire, DE72 2GD, UK

Harlan Study Number: 41203136
Study Title: S-10821:
Daphnia sp., 48-Hour Acute Immobilization Test

The study described was performed in compliance with UK GLP standards (Schedule 1, Good Laboratory Practice Regulations 1999 (SI 1999/3106 as amended by SI 2004/0994)). These Regulations are in accordance with GLP standards published as OECD Principles on Good Laboratory Practice (revised 1997, ENV/MC/CHEM(98)17).

This report fully and accurately reflects the procedures used and data generated. There were no circumstances considered to have affected the integrity of the study or the validity of the data.

Study Director: S J Parr

.....
Date: 17 JAN 2013

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QUALITY ASSURANCE STATEMENT

Harlan Laboratories Ltd., Shardlow Business Park, Shardlow, Derbyshire, DE72 2GD, UK

Harlan Study Number: 41203136
Study Title: S-10821:
Daphnia sp., 48-Hour Acute Immobilization Test

The general facilities are inspected at least once a year and the results are reported to management.

Study-related procedures were audited and inspected. The details of these audits and inspections are given below.

Dates and Types of QA Inspections			**Reported to the Study Director and Test Facility Management**
Date of Inspection	Type of Inspection	Phase Inspected	Report Date
02 August 2011	Verification	General Study Plan	02 August 2011
09 July 2012	Process – based	Test Item Preparation	09 July 2012
10 July 2012	Process – based	Test System Preparation	10 July 2012
10 July 2012	Process – based	Exposure	10 July 2012
18 July 2012	Process – based	Assessment of Response	18 July 2012
02 July 2012	Process – Based	Chemical Analysis	02 July 2012
17 October 2012	Confirmation	Draft Report	17 October 2012

This statement confirms that this final report reflects the raw data and the procedures followed.

Quality Assurance: J. BEVAN

.....

Date: 17 JAN 2013

SIGNATURE OF CONTRIBUTING SCIENTISTS

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Analytical Chemistry: D M Mullee

Harlan Laboratories Ltd., Shardlow Business Park, Shardlow, Derbyshire, DE72 2GD, UK

.....

Date: 17 JAN 2013

SUMMARY

Introduction

A study was performed to assess the acute toxicity of the test item to *Daphnia magna*. The method followed was designed to be compatible with the OECD Guidelines for Testing of Chemicals (April 2004) No 202, "*Daphnia* sp., Acute Immobilisation Test" referenced as Method C.2 of Commission Regulation (EC) No. 440/2008.

Methods

Following a preliminary range-finding test, twenty daphnids (4 replicates of 5 animals) were exposed to Water Accommodated Fractions (WAFs) of the test item over a range of nominal loading rates of 0.10, 0.18, 0.32, 0.56 and 1.0 mg/L for 48 hours at a temperature of approximately 21 °C under static test conditions. The number of immobilized *Daphnia* and any adverse reactions to exposure were recorded after 24 and 48 hours.

Results

The 48-Hour EL^{*}_{50} for the test item to *Daphnia magna* based on nominal loading rates was 0.63 mg/L with 95% confidence limits of 0.55 – 0.72 mg/L loading rate WAF. The Lowest Observed Effect Loading rate was considered to be 0.32 mg/L loading rate WAF. The No Observed Effect Loading rate was 0.18 mg/L loading rate WAF.

Chemical analysis of the test preparations at 0 hours showed measured test concentrations to range from 0.0388 to 0.244 mg/L. Chemical analysis of the test preparations at 48 hours showed a decline in measured test concentrations in the range of less than the to limit of quantitation (LOQ) of the analytical method employed (assessed as 0.0068 mg/L) to 0.0849 mg/L. This decline was considered to be due to the unstable nature of the test item over the test period. Given that an assessment of volatility showed only a 6% loss occurred when a steady stream of compressed air was passed over the test item for a period of 3 hours, it was considered that the decline in measured concentration observed was due to the unstable nature of the test item over the test period rather than volatilization. This was confirmed by the results obtained from a study to determine the hydrolysis as a function of pH and adsorption coefficient (Harlan Study Number: 41203141) which showed that the test item had a half life of 27.0 hours at pH 7.

Given that the toxicity cannot be attributed to a single component or a mixture of components, but to the test item as a whole, the results were based on nominal loading rates only.

*EL = Effective Loading Rate

GENERAL INFORMATION

Schedule

Experimental Starting Date:	17 July 2012
Experimental Completion Date:	11 August 2012

Archiving

Unless instructed otherwise by the Sponsor, all original data and the final report will be retained in the Harlan Laboratories Ltd, Shardlow, UK archives for five years after which instructions will be sought as to further retention or disposal. Further retention or return of the data will be chargeable to the Sponsor.

1 INTRODUCTION AND PURPOSE

This report contains a description of the methods used and results obtained during a study to investigate the acute toxicity of the test item to *Daphnia magna*.

Daphnia magna is a freshwater invertebrate representative of a wide variety of natural habitats, and can therefore be considered as an important non-target organism in freshwater ecosystems.

In view of the difficulties associated with the evaluation of aquatic toxicity of poorly water soluble test items, a modification of the standard method for the preparation of aqueous media was performed. An approach endorsed by several important regulatory authorities in the EU and elsewhere (ECETOC 1996 and OECD 2000), is to expose organisms to a Water Accommodated Fraction (WAF) of the test item in cases where the test item is a complex mixture and is poorly soluble in water and in the permitted auxiliary solvents and surfactants. Using this approach, aqueous media are prepared by mixing the test item with water for a prolonged period. Pre-study work showed a preparation period of 24 hours was sufficient to ensure equilibration between the test item and water phase. At the completion of mixing and following a 1-Hour settlement period, the test item phase is separated by siphon and the test organisms exposed to the aqueous phase or WAF (which may contain dissolved test item and/or leachates from the test item). Exposures are expressed in terms of the original concentration of test item in water at the start of the mixing period (loading rate) irrespective of the actual concentration of test item in the WAF.

1.1 Guidelines / Regulations

This study was designed to be compatible with the procedures indicated by the following internationally accepted guidelines and recommendations:

- OECD Guidelines for Testing of Chemicals (April 2004) No 202 "*Daphnia* sp., Acute Immobilisation Test".
- Method C.2 of Commission Regulation (EC) No. 440/2008.

2 TEST ITEM

The integrity of the supplied data relating to the identity, purity and stability of the test item is the responsibility of the Sponsor. A Certificate of Analysis supplied by the Sponsor is given in Appendix 1.

Identification:	S-10821
Description:	orange colored liquid
Batch:	20334-48
Purity:	see Appendix 1
Expiry / retest date:	2 May 2014
Storage conditions:	room temperature in the dark

3 MATERIALS AND METHODS

3.1 Definitions and Abbreviations

Immobilization:	Those organisms which are not able to swim within 15 seconds after gentle agitation of the test container are considered to be immobile.
EL_{50}:	The calculated loading rate of test item which results in 50% immobilization within the exposure period.
LOEL (Lowest Observed Effect Loading rate):	The lowest loading rate at which a significant toxic effect on the test organisms is observed within the exposure period.
NOEL (No Observed Effect Loading rate):	The highest loading rate at which no significant toxic effect on the test organisms is observed within the exposure period.
Treatment:	Comprises test item treatments and control (test water only).

3.2 Test System

The test was carried out using 1st instar *Daphnia magna* derived from in-house laboratory cultures.

Adult *Daphnia* were maintained in 150 mL glass beakers containing Elendt M7 medium (see Appendix 2) in a temperature controlled room at approximately 20 °C. The lighting cycle was controlled to give a 16 hours light and 8 hours darkness cycle with 20 minute dawn and dusk transition periods. Each culture was fed daily with a mixture of algal suspension (*Desmodesmus subspicatus*) and Tetramin® flake food suspension. Culture conditions ensured that reproduction was by parthenogenesis. Gravid adults were isolated the day before initiation of the test, such that the young daphnids produced overnight were approximately 24 hours old. These young were removed from the cultures and used for testing. The diet and diluent water are considered not to contain any contaminant that would affect the integrity or outcome of the study.

A positive control (Harlan Laboratories Ltd., Study Number: 41203341) used potassium dichromate as the reference item. Details of the positive control are given in Appendix 3. The positive control was conducted between 6 June 2012 and 8 June 2012.

3.3 Test Water

Reconstituted water used for both the range-finding and definitive tests is defined in Appendix 4.

3.4 Procedure

3.4.1 Validation of Mixing Period

Pre-study work was carried out to determine whether stirring for a prolonged period produced significantly higher measured test concentrations in the WAF. A WAF of nominal loading rate of 100 mg/L was prepared, in duplicate, in deionized reverse osmosis water. One loading rate was stirred for a period of 23 hours and the other for a period of 95 hours. After a 1-Hour standing period the mixtures were then removed by siphon and the concentration of the test item in the 100 mg/L loading rate WAF was verified by chemical analysis (see Appendix 5).

3.4.2 Range-finding Tests

Due to the low aqueous solubility and complex nature of the test item, for the purposes of the range-finding test the test item was prepared as a Water Accommodated Fraction (WAF).

The loading rates to be used in the definitive test were determined by preliminary range-finding tests.

In the initial range-finding test *Daphnia magna* were exposed to a series of nominal loading rates of 1.0, 10 and 100 mg/L.

Amounts of test item (10, 40 and 250 mg) were each separately added to the surface of 10, 4 and 2.5 liters of reconstituted water to give the 1.0, 10 and 100 mg/L loading rates respectively. After the addition of the test item, the reconstituted water was stirred by magnetic stirrer using a stirring rate such that a vortex was formed to give a dimple at the water surface. The stirring was stopped after 23 hours and the mixtures allowed to stand for 1 hour. Visual observations made on the WAFs indicated that a significant amount of dispersed test item was present in the water column and hence it was considered justifiable to remove the WAFs by filtering through a glass wool plug (2-4 cm in length). A wide bore glass tube, covered at one end with Nescofilm was submerged into the vessel, sealed end down, to a depth of approximately 5 cm from the bottom of the vessel. A length of Tygon tubing was inserted into the glass tube and pushed through the Nescofilm seal. A glass wool plug was inserted into the opposite end of the tubing and the WAF removed by mid-depth siphoning (the first approximate 75-100 mL discarded) to give the 1.0, 10 and 100 mg/L loading rate WAFs. Microscopic observations of the WAFs were performed after filtering and showed no microscopic undissolved particles of test item.

Due to significant immobilization in the preliminary range-finding test, a second range-finding test was conducted in which *Daphnia magna* were exposed to a series of nominal loading rates of 0.0010, 0.10, 0.10 and 1.0 mg/L.
An amount of test item (10 mg) was added to the surface of 10 liters of reconstituted water to give the 1.0 mg/L loading rate. After the addition of the test item, the reconstituted water was stirred by magnetic stirrer using a stirring rate such that a vortex was formed to give a dimple at the water surface. The stirring was stopped after 23 hours and the mixture allowed to stand for 1 hour. A wide bore glass tube, covered at one end with Nescofilm was submerged into the

vessel, sealed end down, to a depth of approximately 5 cm from the bottom of the vessel. A length of Tygon tubing was inserted into the glass tube and pushed through the Nescofilm seal. Microscopic inspection of the WAF showed no micro-dispersions or undissolved test item to be present. The aqueous phase or WAF was removed by mid-depth siphoning (the first approximate 75-100 mL discarded) to give the 1.0 mg/L loading rate WAF. Serial dilutions were then performed in reconstituted water to give the further test concentrations of 0.001, 0.010 and 0.10 mg/L loading rate WAF.

In the range-finding tests 10 daphnids were placed in each test and control vessel and maintained in a temperature controlled room at approximately 21 °C with a photoperiod of 16 hours light and 8 hours darkness for a period of 48 hours with 20 minute dawn and dusk transition periods. Each 250 mL test and control vessel contained 200 mL of test media and was covered to reduce evaporation. After 24 and 48 hours the number of immobilized *Daphnia magna* were recorded.

The control group was maintained under identical conditions but not exposed to the test item.

A sample of each loading rate WAF was taken for chemical analysis at 0 and 48 hours in order to determine the stability of the test item under test conditions. All samples were stored at approximately -20 °C prior to analysis. Only concentrations within the range to be used for the definitive test were analyzed.

3.4.3 Definitive Test

Based on the results of the range-finding tests the following loading rates were assigned to the definitive test: 0.10, 0.18, 0.32, 0.56 and 1.0 mg/L.

3.4.3.1 Experimental Preparation

An amount of test item (10 mg) was added to the surface of 10 liters of reconstituted water to give the 1.0 mg/L loading rate. After the addition of the test item, the reconstituted water was stirred by magnetic stirrer using a stirring rate such that a vortex was formed to give a dimple at the water surface. The stirring was stopped after 23 hours and the mixture allowed to stand for 1 hour. A wide bore glass tube, covered at one end with Nescofilm was submerged into the vessel, sealed end down, to a depth of approximately 5 cm from the bottom of the vessel. A length of Tygon tubing was inserted into the glass tube and pushed through the Nescofilm seal. Microscopic inspection of the WAF showed undissolved test item to be present so therefore it was justifiable to filter the WAF through a glass wool plug. The aqueous phase or WAF was removed by mid-depth siphoning (the first approximate 75-100 mL discarded) to give the 1.0 mg/L loading rate WAF. Microscopic inspection of the WAF after siphoning through glass wool showed no micro-dispersions or undissolved test item to be present. Aliquots of the 1.0 mg/L loading rate WAF (200, 360, 640 and 1120 mL) were each separately added to a final volume of 2 liters of reconstituted water to give the 0.10, 0.18, 0.32 and 0.56 mg/L loading rate WAFs respectively.

The concentration and stability of the test item in the test preparations were verified by chemical analysis at 0 and 48 hours (see Appendix 6).

3.4.3.2 Exposure Conditions

As in the range-finding tests 250 mL glass jars containing approximately 200 mL of test preparation were used. At the start of the test 5 daphnids were placed in each test and control vessel at random, in the test preparations. Four replicate test and control vessels were prepared. The test vessels were then covered to reduce evaporation and maintained in a temperature controlled room at approximately 21 °C with a photoperiod of 16 hours light and 8 hours darkness with 20 minute dawn and dusk transition periods with a light intensity ranging from 667 to 706 lux. The daphnids were not individually identified, received no food during exposure and the test vessels were not aerated.

The control group was maintained under identical conditions but not exposed to the test item.

The test preparations were not renewed during the exposure period. Any immobilization or adverse reactions to exposure were recorded at 24 and 48 hours after the start of exposure. The criterion of effect used was that *Daphnia* were considered to be immobilized if they were unable to swim for approximately 15 seconds after gentle agitation.

3.4.3.3 Physico-Chemical Measurements

Water temperature was recorded daily throughout the test. Dissolved oxygen concentrations and pH were recorded at the start and termination of the test. The pH and dissolved oxygen concentration were measured using a Hach HQ30d Flexi handheld meter whilst the temperature was measured using a Hanna Instruments HI 93510 digital thermometer.

3.4.3.4 Vortex Depth Measurements

The vortex depth was recorded at the start and end of the mixing period.

3.4.3.5 Chemical Analysis of Test Loading Rates

Water samples were taken from the control and each loading rate WAF test group (replicates R_1 – R_4 pooled) at 0 and 48 hours for quantitative analysis. Samples were stored at approximately -20 °C prior to analysis.

Duplicate samples were taken and stored at approximately -20 °C for further analysis if necessary.

Samples at the No Observed Effect Loading Rate and above only were analyzed.

The method of analysis, recovery and test preparation analyses are described in Appendix 6.

3.4.3.6 Evaluation of Data

The EL^{*}_{50} values and associated confidence limits at 24 and 48 hours were calculated by the trimmed Spearman-Karber method (Hamilton *et al*, 1977) using the ToxCalc computer software package (ToxCalc, 1999)

When only one partial response is shown the trimmed Spearman-Karber method is appropriate.

*EL = Effective Loading Rate

4 RESULTS

4.1 Validation of Mixing Period

Pre-study investigational work (see Appendix 5) indicated that there was no significant increase in the amount of test item by extending the preparation period for longer than 24 hours. Therefore, for the purpose of testing the test item was prepared using a stirring period of 23 hours followed by a 1-Hour settlement period.

4.2 Range-finding Tests

Cumulative immobilization data from the exposure of *Daphnia magna* to the test item during the range-finding tests are given in Table 1 and 2.

In the initial range-finding test immobilization was observed in all of the test concentrations. In the second range-finding test no immobilization was observed at 0.0010, 0.010 and 0.10 mg/L loading rate WAF. However, immobilization was observed at 1.0 mg/L loading rate WAF.

Based on this information loading rates of 0.10, 0.18, 0.32, 0.56 and 1.0 mg/L, using a stirring period of 23 hours followed by a 1-Hour standing period, were selected for the definitive test.

Chemical analysis of the 0.10 and 1.0 mg/L loading rate WAF preparations at 0 hours (see Appendix 6) showed measured concentrations of 0.037 and 0.46 mg/L respectively were obtained. A decline in measured concentration was observed at 48 hours to 0.014 and 0.22 mg/L for the 0.10 and 1.0 mg/L loading rates respectively, indicating that the test item was unstable over the test period.

4.3 Definitive Test

4.3.1 Immobilization Data

Cumulative immobilization data from the exposure of *Daphnia magna* to the test item during the definitive test are given in Table 3. The relationship between percentage immobilization and concentration at 24 and 48 hours is given in Figure 1 and Figure 2.

Analysis of the immobilization data by the trimmed Spearman-Karber method (Hamilton *et al*, 1977) at 24 and 48 hours based on the nominal loading rates gave the following results:

Time (h)	EL^{*}_{50} (mg/L Loading Rate WAF)	95% Confidence limits (mg/L Loading Rate WAF)
24	0.71	0.65 – 0.76
48	0.63	0.55 – 0.72

*EL = Effective Loading Rate

A single immobilized daphnid was observed at 0.18 mg/L loading rate WAF after 48 hours exposure. This was considered to be due to natural causes rather than a true toxic response as less than 10 % immobilization occurred.

The No Observed Effect Loading rates after 24 and 48 hours exposure were 0.32 and 0.18 mg/L loading rate WAFs respectively. Correspondingly the lowest observed effect loading rates after 24 and 48 hours were 0.56 and 0.32 mg/L respectively.

Due to the unsuitable nature of the data it was not possible to calculate the slope and error of response curve at 24 and 48 hours.

4.3.2 Physico-Chemical Measurements

The results of the physico-chemical measurements are given in Appendix 7.

Temperature was maintained at approximately 21 °C throughout the test, while there were no treatment related differences for oxygen concentration or pH.

The oxygen concentration in some of the test vessels at 0 hours was observed to have an air saturation value (ASV) in excess of 100%. This was considered to be due to the presence of microscopic air bubbles in the media super-saturating the diluent and was considered not to have had an impact on the outcome or integrity of the test as no adverse effects were observed in the control group.

4.3.3 Vortex Depth Measurements

The vortex depth was recorded at the start and end of the mixing period and was observed to be a dimple at the water surface on each occasion (see Table 4).

4.3.4 Observations on Test Item Solubility

Observations on the test media were carried out during the mixing and testing of the WAF.

At the start of the mixing period the 1.0 mg/L loading rate was observed to be a clear colorless water column with oily globules of test item floating on the surface. After 23 hours stirring and a 1-Hour standing period the 1.0 mg/L loading rate was observed to remain as at the start of the stirring period. Microscopic inspection of the WAF showed no micro-dispersions or undissolved test item to be present after filtration through glass wool. After siphoning and for the duration of the test, the 0.10, 0.18, 0.32, 0.56 and 1.0 mg/L loading rates were observed to be clear, colorless solutions.

4.3.5 Chemical Analysis of Test Loading Rates

Chemical analysis of the test preparations at 0 hours (see Appendix 6) showed measured test concentrations to range from 0.0388 to 0.244 mg/L. Chemical analysis of the test preparations at 48 hours showed a decline in measured test concentration in the range of less than the limit of quantitation (LOQ) of the analytical method employed (assessed as 0.0068 mg/L) to 0.0849 mg/L. Given that an assessment of volatility showed only a 6% loss occurred when a steady stream of compressed air was passed over the test item for a period of 3 hours, it was considered that the decline in measured concentration observed was due to the unstable nature of the test item over the test period rather than volatilization. This was confirmed by the results obtained from a study to determine the hydrolysis as a function of pH and adsorption coefficient (Harlan Study Number: 41203141) which showed that the test item had a half life of 27.0 hours at pH 7.

Given that the toxicity cannot be attributed to a single component or a mixture of components, but to the test item as a whole, the results were based on nominal loading rates only.

5 CONCLUSION

The acute toxicity of the test item to the freshwater invertebrate *Daphnia magna* has been investigated and gave a 48-Hour EL^{*}_{50} value of 0.63 mg/L loading rate WAF with 95% confidence limits of 0.55 – 0.72 mg/L loading rate WAF. The Lowest Observed Effect Loading rate at 48 hours was considered to be 0.32 mg/L loading rate WAF. The No Observed Effect Loading rate at 48 hours was 0.18 mg/L loading rate WAF.

6 REFERENCES

ENVIRONMENT DIRECTORATE, ORGANISATION FOR ECONOMIC CO-OPERATION AND DEVELOPMENT (OECD) (2000) Guidance Document on Aquatic Toxicity Testing of Difficult Substances and Mixtures.

EUROPEAN CENTRE FOR ECOTOXICOLOGY AND TOXICOLOGY OF CHEMICALS (ECETOC) Monograph No. 26 (1996) Aquatic Toxicity Testing of Sparingly Soluble, Volatile and Unstable Substances.

HAMILTON, M A, RUSSO, R C AND THURSTON, R V (1977) Trimmed Spearman-Karber Method for Estimating Median Lethal Concentration in Toxicity Bioassays. Environ Sci Technol 11, 714-719.

TOXCALC VERSION 5.0.23C (1999), Tidepool Scientific Software, McKinleyville, CA 95519, USA.

* EL = Effective Loading rate

TABLES

Table 1 Cumulative Immobilization Data in the Initial Range-finding Test

Nominal Loading Rate (mg/L)	Cumulative Immobilized *Daphnia* (Initial Population: 10 Per Replicate)	
	24 Hours	48 Hours*
Control	0	-
1.0	10	-
10	10	-
100	10	-

* Test was terminated after 24 hours due to complete immobilization in all test concentrations.

Table 2 Cumulative Immobilization in the Second Range-finding Test

Nominal Loading Rate (mg/L)	Cumulative Immobilized *Daphnia* (Initial Population: 10 Per Replicate)	
	24 Hours	48 Hours
Control	0	0
0.0010	0	0
0.010	0	0
0.10	0	0
1.0	10	10

Table 3 Cumulative Immobilization Data in the Definitive Test

Nominal Loading Rate (mg/L)	Cumulative Immobilized *Daphnia* (Initial Population: 5 Per Replicate)											
	24 Hours						48 Hours					
	R_1	R_2	R_3	R_4	Total	%	R_1	R_2	R_3	R_4	Total	%
Control	0	0	0	0	0	0	0	0	0	0	0	0
0.10	0	0	0	0	0	0	0	0	0	0	0	0
0.18	0	0	0	0	0	0	0	1	0	0	1†	5
0.32	0	0	0	0	0	0	0	2	0	1	3	15
0.56	0	2	0	0	2	10	0	2	0	0	2	10
1.0	5	5	5	5	20	100	5	5	5	5	20	100

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$R_1 - R_4$ = Replicates 1 to 4
† Single immobilized daphnid considered to be due to natural causes rather than a true toxic effect as less than 10% immobilization occurred.

Table 4 Vortex Depth Measurements at the Start and End of the Mixing Period

	Nominal Loading Rate (mg/L)			
	Control		1.0	
	*	+	*	+
Height of Water Column (cm)	14.5	14.5	26.7	26.7
Depth of Vortex (cm)	~0.2	~0.2	~0.2	~0.2
Observation of Vortex	Dimple present	Dimple present	Dimple present	Dimple present

* = Start of mixing period
+ = End of mixing period

FIGURES

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Figure 1 Loading Rate Response Curve after 24 Hours

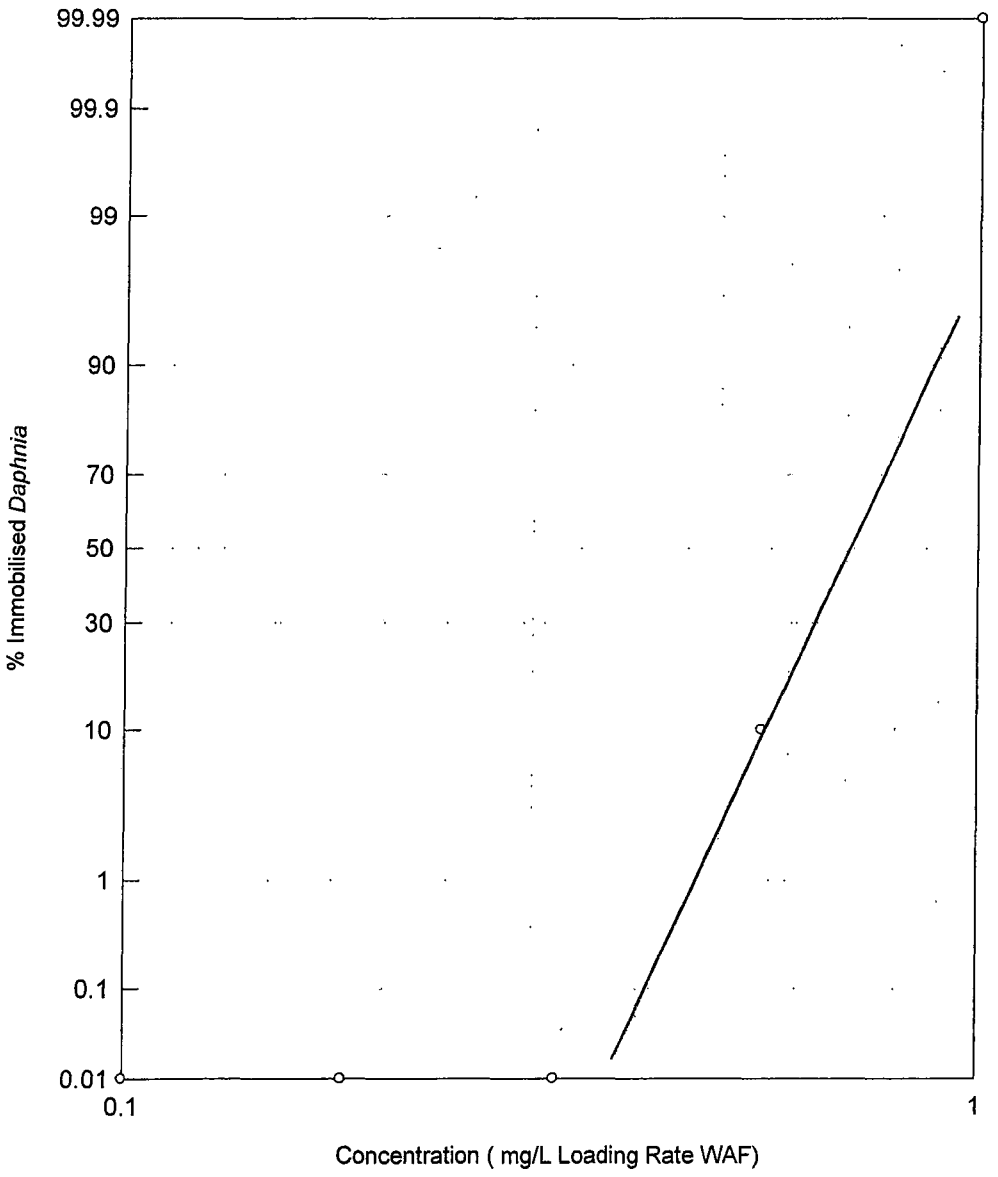
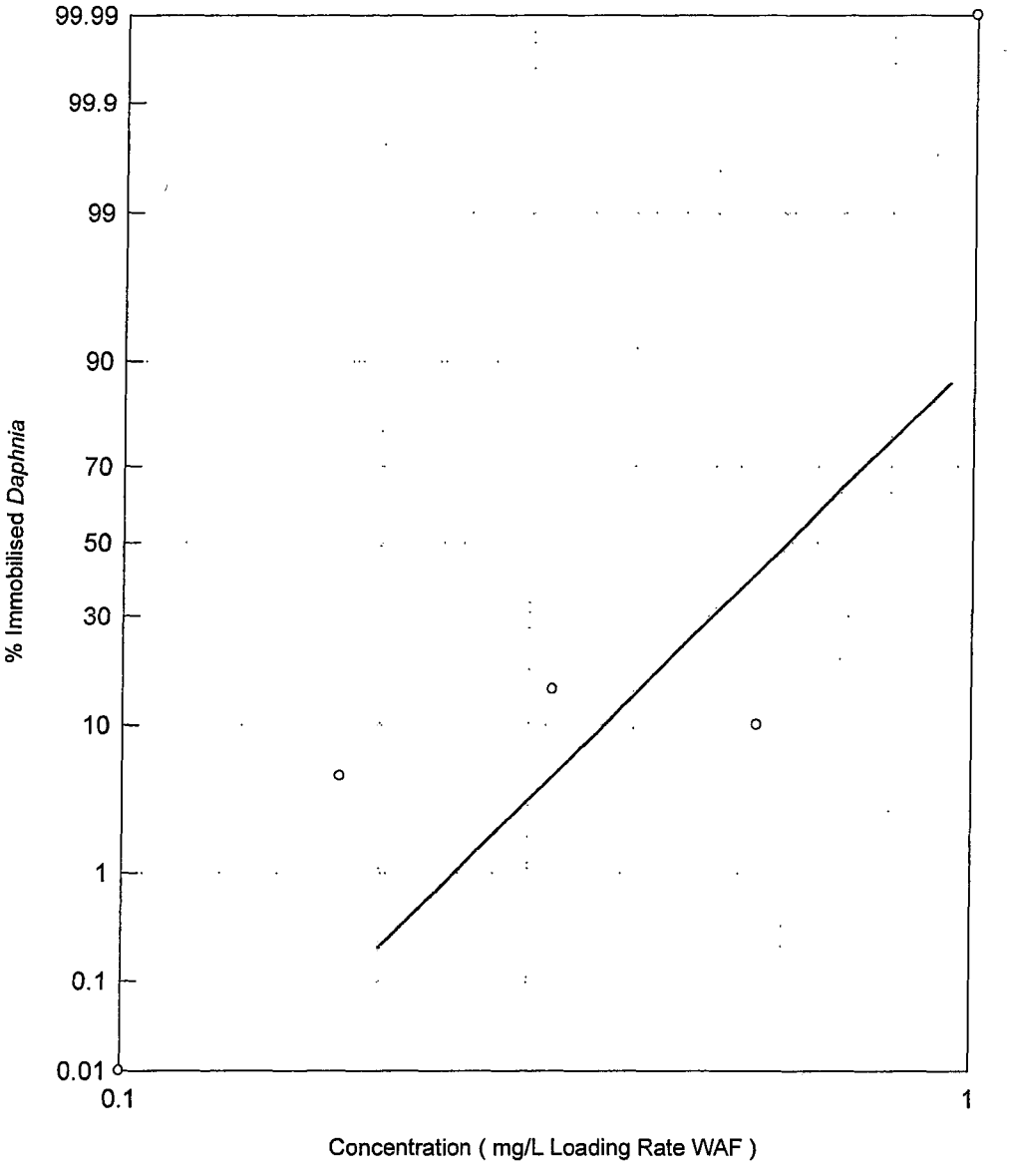


Figure 2 Loading Rate Response Curve after 48 Hours



APPENDICES

Appendix 1 Certificate of Analysis

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CYTEC

Cytec Industries Inc.
Product Sustainability & Regulatory Affairs Dept.
5 Garret Mountain Plaza
Woodland Park, NJ 07424

May 18, 2012

Harlan Laboratories Ltd
Shardlow Business Park
London Road
Shardlow Derbyshire DE72 2GD
United Kingdom

Attention: Test Item Control

Reference: Certificate of Composition (CONFIDENTIAL)

Test Material: **S-10821** (CT 962-11)

Composition*:

Component/CAS No	%
Carbonodithioic acid, O-pentyl S-propenyl ester CAS# 2956-12-9	~57.1
Carbonodithioic acid, O-(2-methylbutyl) S-2-propen-1-yl ester CAS# 1311195-87-5	~29.7
Pentyl Alcohol CAS# 71-41-0	~3.9
2-Methyl-1-butanol CAS# 137-32-6	~1.3%
Water 7732-18-5	~0.05%
Unidentified Impurities No CAS# Assigned	~8

Storage: **Stable at Room Temperature; see SDS for complete information**

Expiration Date: **2 years from date of receipt**

Patti Vernon
Cytec Industries Inc.
Phone: 973-357-3375
Email: Patricia.Vernon@cytec.com

Appendix 2 Reconstituted Water – Elendt M7 Medium

Solution		Concentration of stock solution (mg/L)
(I)	H_3BO_3	57190
(II)	$MnCl_2.4H_2O$	7210
(III)	LiCl	6120
(IV)	RbCl	1420
(V)	$SrCl_2.6H_2O$	3040
(VI)	NaBr	320
(VII)	$Na_2MoO_4.2H_2O$	1260
(VIII)	$CuCl_2.2H_2O$	335
(IX)	$ZnCl_2$	260
(X)	$CoCl_2.6H_2O$	200
(XI)	KI	65
(XII)	Na_2SeO_3	43.8
(XIII)	NH_4VO_3	11.5
(XIV)	$Na_2EDTA.2H_2O$	5000
	$FeSO_4.7H_2O$	1991

An aliquot (dependant on the volume of medium required) of each stock solution was added to a final volume of deionized reverse osmosis water to give stock solution A and stored at approximately 21 - 25 °C.

Macro Nutrient Stock Solutions

Solution		Concentration of stock solution (g/L)
(XV)	$CaCl_2.2H_2O$	293.80
(XVI)	$NaHCO_3$	64.80
(XVII)	$MgSO_4.7H_2O$	246.60
(XVIII)	$Na_2SiO_3.9H_2O$	50.00
(XIX)	KCl	58.00
(XX)	$NaNO_3$	2.74
(XXI)	K_2HPO_4	1.84
(XXII)	KH_2PO_4	1.43

Vitamin Nutrients

Solution		Concentration of stock solution (mg/L)
(XXIII)	Thiamine hydrochloride	750
	Cyanocobalamine (vitamin B_{12})	10
	D(+) biotin (vitamin H)	7.5

The final medium was prepared by adding an aliquot of stock solution A along with aliquots of each individual Macro Nutrient Stock Solution and an aliquot of the vitamin nutrient to the required amount (final volume) of deionized reverse osmosis water.

The pH of the prepared media was 8.0 ± 0.2 and stored at approximately 21 °C.

Appendix 3 Positive Control

A positive control (Harlan Laboratories Ltd., Study Number: 41203341) used potassium dichromate as the reference item at concentrations of 0.32, 0.56, 1.0, 1.8 and 3.2 mg/L.

Exposure conditions for the positive control were similar to those in the definitive test.

Analysis of the immobilization data by the geometric mean method at 24 hours and the trimmed Spearman-Karber method (Hamilton *et al* 1977*) at 48 hours based on the nominal test concentrations gave the following results:

Time (h)	EC_{50} (mg/L)	95% Confidence limits (mg/L)
24	0.75	0.56 - 1.0
48	0.45	0.42 - 0.48

The No Observed Effect Concentrations after 24 and 48 hours were 0.56 and 0.32 mg/L respectively. The No Observed Effect Concentration is based upon zero immobilization at this concentration.

The results from the positive control with potassium dichromate were within the normal range for this reference item.

* HAMILTON, M A, RUSSO, R C AND THURSTON, R V (1977) Trimmed Spearman-Karber Method for Estimating Median Lethal Concentration in Toxicity Bioassays. Environ Sci Technol 11, 714-719.

Appendix 4 Reconstituted Water

i) Stock Solutions

a)	$CaCl_2.2H_2O$	11.76 g/L
b)	$MgSO_4.7H_2O$	4.93 g/L
c)	$NaHCO_3$	2.59 g/L
d)	KCl	0.23 g/L

ii) Preparation

An aliquot (25 mL) of each of solutions a-d was added to each liter (final volume) of deionized water with a conductivity of <5 $\mu S\ cm^{-1}$. The reconstituted water had a pH of 7.8 ± 0.2 adjusted (if necessary) with NaOH or HCl and was aerated until the dissolved oxygen concentration was approximately air-saturation value.

The reconstituted water had an approximate theoretical total hardness of 250 mg/L as $CaCO_3$.

Appendix 5 Validation of Mixing Period

Pre-study work was carried out to determine whether stirring for a prolonged period produced significantly higher levels of total organic carbon, as an indicator of soluble organic items, in the WAF. A WAF of a nominal loading rate of 100 mg/L was prepared in duplicate in deionized reverse osmosis water and stirred using a stirring rate such that a vortex was formed to give a slight dimple at the water surface. One loading rate was stirred for a period of 23 hours and the other for a period of 95 hours. After a 1-Hour standing period the mixtures were then removed by siphon and samples taken for chemical analysis.

The results are summarized as follows:

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Nominal Loading Rate (mg/L)	Time (Hours)	
	24	96
	mg/L	mg/L
Control	<LOQ*	<LOQ*
100	3.63	3.47

It is evident from this work that increasing the stirring period did not significantly increase the amount of dissolved test item in the WAF and so preparation of the WAF was maintained at 24 hours.

* LOQ = Limit of Quantitation which was considered to be 1.0 mg C/L.

Appendix 6 Analytical Investigations

Analytical Investigations

CONTENTS

1 MATERIALS AND METHODS

1.1 Introduction

The test item concentration in the test samples was determined by gas chromatography (GC) using an external standard. The test item gave a chromatographic profile consisting of two peaks.

The method was developed by the Department of Analytical Services, Harlan Laboratories Ltd, Shardlow, UK.

1.2 Test Item

The test item is described in the biological part of this study.

1.3 Analytical Standard

The test item described in the biological part of this study was also used as the analytical standard.

1.4 Analytical Procedure

1.4.1 Storage

The samples were stored at approximately -20 °C prior to analysis.

1.4.2 Reagents and Solvents

Water: prepared using an ELGA Pure Lab Option R-15 water purification system.

Test medium: as described in the biological part of this study.

Solvents: methanol, Fisher Scientific, HPLC grade.
hexane, Fisher Scientific, GLC pesticide residue grade.

Reagents: sodium chloride, Fisher Scientific, laboratory reagent grade.

1.4.3 Preparation of Calibration Standards

The test item (nominal 100 mg) was dissolved in hexane (100 mL) to prepare a stock solution with a nominal concentration of 1000 mg/L. This stock solution was further diluted with hexane to obtain a nominal 5 mg/L calibration standard. A duplicate calibration standard was similarly prepared at 5 mg/L. These duplicate calibration standards were used to determine the recovery and test sample concentrations.

1.4.4 Preparation of Linearity Standards

The test item (nominal 100 mg) was dissolved in methanol (100 mL) to prepare a stock solution with a nominal concentration of 1000 mg/L. Defined volumes of this stock solution were diluted with methanol to obtain standards in the range of 0.10 to 10 mg/L. A second standard was similarly prepared at a nominal concentration of 5 mg/L. These standards were used to evaluate the linearity of the analytical system.

1.4.5 Preparation of Spiked Recovery Samples

To demonstrate the validity of the analytical procedure, volumes of test medium were spiked with the test item and its recovery was assessed. The test item (nominal 100 mg) was initially dissolved in methanol to prepare a stock solution with a concentration of 1000 mg/L. This stock solution was further diluted with methanol to produce solutions of 5.0 and 50 mg/L. Defined volumes of these stock solutions were diluted with test medium to obtain spiked recovery samples at concentrations of 0.050 and 0.50 mg/L. Five replicates at each concentration level were prepared and subjected to the same treatment as the test samples. In addition, test medium without the addition of the test item (synthetic control) was also analyzed.

1.4.6 Preparation of Test Samples

The test samples were thawed prior to analysis.

The test samples (150 mL) plus 30 g of sodium chloride were extracted directly with 15 mL of hexane in the same bottle. An aliquot of the hexane layer was taken for analysis.

See Table 1 for more information on preparation volumes.

1.4.7 Instrumental Parameters

Gas chromatograph:	7890A, with autosampler
Detector:	FID
Column:	Restek, Rxi-1ms (15 m x 0.25 mm i.d.; 1 µm film thickness)
Carrier gas:	nitrogen
Mode:	constant pressure
Pressure:	15 psi
Oven temperature Program:	oven: 40 °C, for 1 minute with 20 °C/minute to 250 °C, for 1 minute
Injector temperature:	300 °C
Injection mode:	splitless
Injection volume:	1 µL
Inlet purge time:	1 minute
Detector temperature:	300 °C
Retention times:	approximately 9.0 and 9.4 minutes

1.4.8 Calculations

A linear response was obtained during the validation of the analytical method, so for the definitive test samples a single-point calibration was performed using duplicate calibration standards. The mean peak areas of each calibration standard were corrected to a nominal concentration and the mean value taken.

The concentration "x" of the test item in an injected sample was calculated by the following equation:

$$x = \frac{R_{spl.}\, N}{R_{cal\ std}} \qquad (1)$$

where
- x: concentration of the test item in injected sample [mg/L]
- R_{spl}: mean peak area response of the test item in injected sample [counts]
- $R_{cal\ std}$: mean peak area response for the calibration standard, corrected to nominal concentration [counts]
- N: nominal calibration standard concentration (mg/L)

The concentration of the test item in the test samples was calculated using the following equation:

$$c = x \cdot F \qquad (2)$$

where c: concentration of the test item in the test sample [mg/L]
x: concentration of the test item in the injected sample solution [mg/L]
F: sample preparation factor

The recovery rate of the spiked recovery samples was calculated using the following equation:

$$R = \frac{c}{c_{fort}} \cdot 100 \qquad (3)$$

where R: recovery rate [%]
c: determined concentration of the test item in the spiked recovery sample [mg/L]
c_{fort}: fortified concentration of the test item in the spiked recovery sample [mg/L]

2 RESULTS

2.1 Range-Finding Test

The analytical methodology was not validated prior to the analysis of range-finding samples; full validation was later conducted specifically over the concentration range to be used in the definitive test. The results for the range-finding test samples are summarized in Table 3.

2.2 Definitive Test

2.2.1 Validation of the Analytical Method

Specificity
The biological control samples and an analyzed analytical blank showed no significant interfering response at the retention time of the test item. The standard solutions contained a peak specific for the test item whose area changed accordingly with known concentration, hence the specificity of the method by retention time was confirmed.

Linearity
The calibration data for the calibration standards of the test item is given in Table 2, and Figure 1. The data was found to have a linear correlation within the calibration range of 0 to 9.1 mg/L. The R^2 fit of the calibration curve to the data was 0.9969, and was considered to be acceptable.

Accuracy (Recovery) and Precision
A set of recovery samples accurately fortified at relevant a concentration of test item was prepared five-fold and analyzed. The results obtained for the concentration of the test item in the recovery samples are presented in Table 4.

The method was considered to be sufficiently accurate and precise for the purposes of this test. The test sample results were not corrected for recovery.

Limit of Quantitation (LOQ)
The limit of quantitation (LOQ) was determined by calculating the sample concentration that gave a peak equivalent to ten times the baseline noise. Using this method the limit of quantitation was determined to be 0.0068 mg/L.

2.2.2 Test Samples

The results obtained for the concentration of the test item found in the test samples are presented in Table 5.

Typical chromatograms are shown in Figures 2 to 4.

3 DISCUSSION

The detection system was found to have acceptable linearity. The analytical procedure was found to have acceptable recoveries of test item in test medium. The method of analysis was validated and proven to be suitable for use.

TABLES

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Table 1 Preparation of Test Samples

Nominal Concentration of Test Item Loading Rate WAF [mg/L]	Sample Volume [mL]	Final Volume [mL]	Sample Preparation Factor
Control	150	15	0.10
0.18	150	15	0.10
0.32	150	15	0.10
0.56	150	15	0.10
1.0	150	15	0.10

Table 2 Linearity Data

Concentration of Test Item [mg/L]	Area [counts]
0	0
0.0907	5.463×10^3
0.907	5.900×10^4
3.63	2.458×10^5
4.54	3.190×10^5
4.75	3.309×10^5
9.07	6.886×10^5

Table 3 Results for Range-Finding Samples

Time point [hours]	Nominal Concentration of Test Item in Range-Finding Sample Loading Rate WAF c_{nom} [mg/L]	Measured Concentration of Test Item in Sample Vial x [mg/L]	Sample Preparation Factor F	Determined Concentration of Test Item in Range-Finding Sample c [mg/L]
0	0.10	0.368	0.10	0.0368
	1.0	4.62	0.10	0.462
48	0.10	0.144	0.10	0.0144
	1.0	2.21	0.10	0.221

Table 4 Results for Spiked Recovery Samples

Nominal Concentration of Test Item c_{nom} [mg/L]	Fortified Concentration of Test Item in the Spiked Sample c_{fort} [mg/L]	Measured Concentration of Test Item in the Sample Vial x [mg/L]	Sample Preparation Factor F	Determined Concentration of Test Item in the Spiked Sample c [mg/L]	Analytical Recovery Rate R [%]	Precision (Relative Standard Deviation of Recovery) [%]
0.050	0.0507	0.412	0.10	0.0412		
		0.414	0.10	0.0414		
		0.440	0.10	0.0440	84	2.9
		0.436	0.10	0.0436		
		0.427	0.10	0.0427		
0.50	0.507	4.58	0.10	0.458		
		4.72	0.10	0.472		
		4.68	0.10	0.468	91	2.3
		4.52	0.10	0.452		
		4.47	0.10	0.447		
Acceptance Target					80-120	<10

The tabulated values are rounded results obtained by calculation using the exact raw data.

Table 5 Results for Test Samples

Time point [hours]	Nominal Concentration of Test Item in Test Sample Loading Rate WAF c_{nom} [mg/L]	Measured Concentration of Test Item in Sample Vial x [mg/L]	Sample Preparation Factor F	Determined Concentration of Test Item in Test Sample c [mg/L]
0	Control	<LOQ	0.10	<LOQ
	0.18	0.388	0.10	0.0388
	0.32	0.727	0.10	0.0727
	0.56	1.22	0.10	0.122
	1.0	2.44	0.10	0.244
48	Control	<LOQ	0.10	<LOQ
	0.18	<LOQ	0.10	<LOQ
	0.32	0.160	0.10	0.0160
	0.56	0.482	0.10	0.0482
	1.0	0.849	0.10	0.0849

LOQ = Limit of Quantitation

The tabulated values are rounded results obtained by calculation using the exact raw data.

FIGURES

Figure 1 Linearity

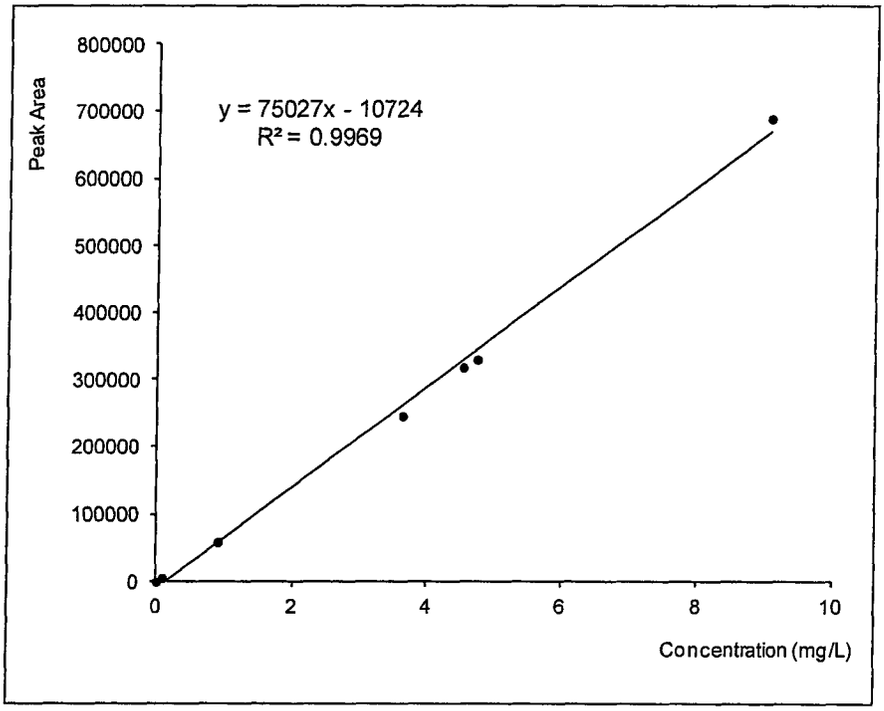

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Figure 2 Chromatogram of Calibration Solution (5.0 mg/L)

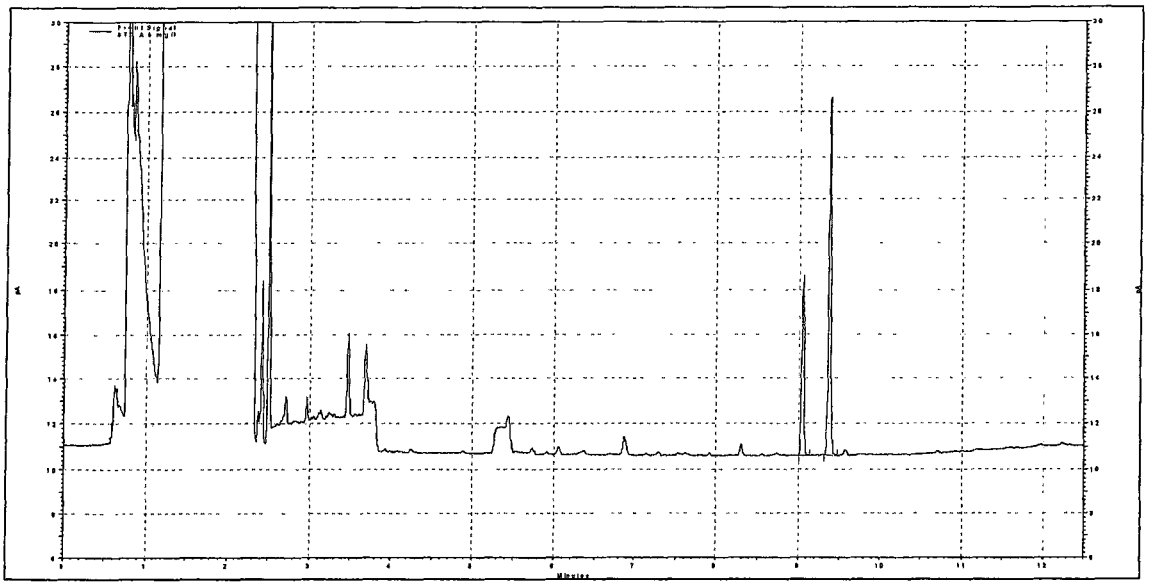


Figure 3 Chromatogram of Biological Control Sample

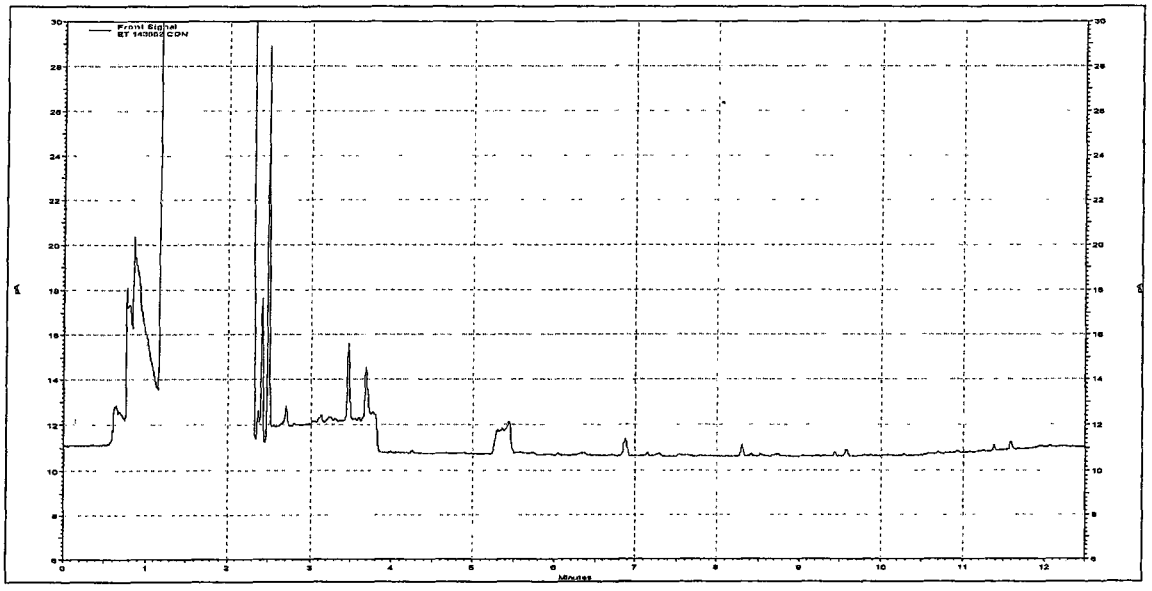
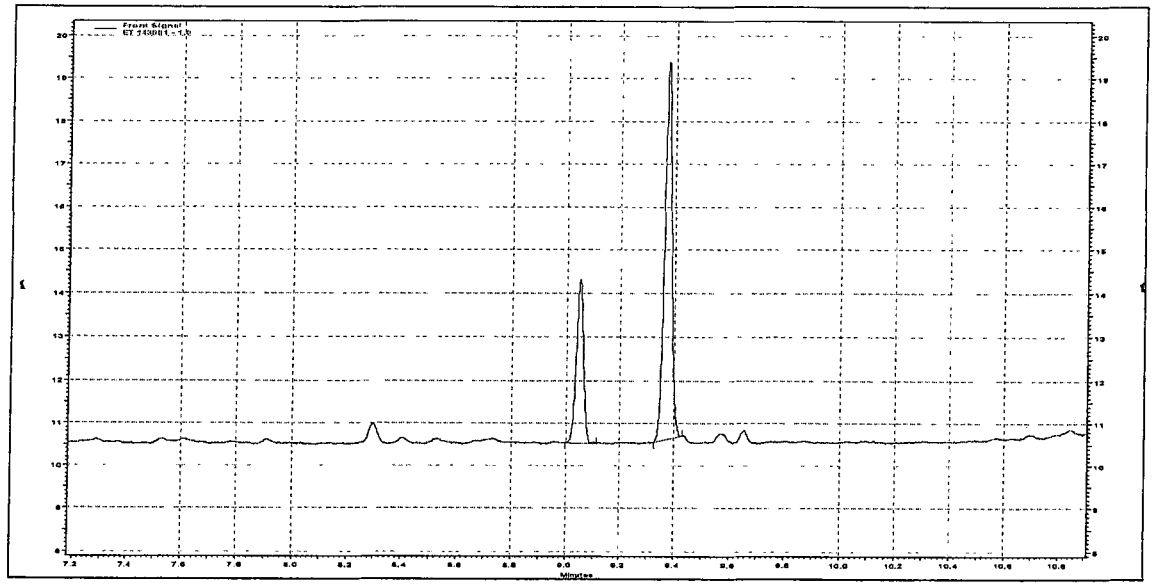


Figure 4 Chromatogram of Test Sample (1.0 mg/L Loading Rate WAR)



Appendix 7 Physico-Chemical Measurements

Nominal Concentration (mg/L Loading Rate WAF)		0 Hours				24 Hours	48 Hours			
		pH	mg O_2/L	%ASV*	T °C	T °C	pH	mg O_2/L	%ASV*	T °C
Control	R_1	8.1	9.0	99	20	22	8.5	8.8	99	21
	R_2	8.0	9.0	99	20	21	8.4	8.8	99	21
	R_3	8.0	9.0	99	20	22	8.4	8.8	99	21
	R_4	8.0	9.0	99	20	21	8.4	8.8	99	21
0.10	R_1	8.0	9.0	101	21	22	8.3	8.7	98	21
	R_2	8.0	9.0	101	21	21	8.3	8.6	97	21
	R_3	8.0	8.9	100	21	22	8.3	8.6	97	21
	R_4	7.9	8.9	100	21	21	8.3	8.6	97	21
0.18	R_1	8.0	8.9	100	21	22	8.3	8.7	98	21
	R_2	8.0	8.9	100	21	21	8.3	8.7	100	22
	R_3	8.0	8.9	100	21	22	8.3	8.7	98	21
	R_4	8.0	8.9	100	21	21	8.3	8.7	98	21
0.32	R_1	8.1	8.9	100	21	22	8.3	8.7	98	21
	R_2	8.1	8.9	100	21	21	8.3	8.7	98	21
	R_3	8.1	8.9	100	21	22	8.3	8.7	98	21
	R_4	8.1	8.9	100	21	21	8.3	8.6	97	21
0.56	R_1	8.2	9.0	101	21	22	8.3	8.6	97	21
	R_2	8.2	8.9	100	21	21	8.4	8.7	98	21
	R_3	8.3	8.9	100	21	21	8.4	8.7	98	21
	R_4	8.3	8.9	100	21	21	8.4	8.7	98	21
1.0	R_1	8.4	8.8	101	22	21	8.4	8.7	98	21
	R_2	8.4	8.8	101	22	21	8.4	8.6	97	21
	R_3	8.5	8.8	101	22	21	8.5	8.7	98	21
	R_4	8.5	8.8	101	22	21	8.5	8.7	98	21

*ASV = Dissolved oxygen concentration expressed as a percentage of Air Saturation Value
$R_1 - R_4$ = Replicates 1 to 4

Appendix 8 Monitoring Authority Statement of GLP Compliance

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Department of Health

THE DEPARTMENT OF HEALTH OF THE GOVERNMENT OF THE UNITED KINGDOM

GOOD LABORATORY PRACTICE

STATEMENT OF COMPLIANCE IN ACCORDANCE WITH DIRECTIVE 2004/9/EC

TEST FACILITY

Harlan Laboratories Ltd
Shardlow Business Park
London Road
Shardlow
Derby, DE27 2GD

TEST TYPE(S)

Analytical/Clinical
Chemistry
Environmental Toxicity
Environmental Fate
Mutagenicity
Phys/Chem. Tests
Toxicology

DATE OF INSPECTION

10 July 2012

An inspection for compliance with the Principles of Good Laboratory Practice was carried out at the above test facility as part of the UK Good Laboratory Practice Compliance Monitoring Programme.

This statement confirms that, on the date of issue, the UK Good Laboratory Practice Monitoring Authority were satisfied that the above test facility was operating in compliance with the OECD Principles of Good Laboratory Practice.

This statement constitutes a Good Laboratory Practice Instrument (as defined in the UK Good Laboratory Practice Regulations 1999).

7/9/12

Dr. Andrew J. Gray
Head, UK GLP Monitoring Authority

MHRA

ORIGIN ID: SXPA (973) 357-3482
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CYTEC INDUSTRIES
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